macrophages[17]. Analysis of the immune responses of *mg*^{3J} animals, which lack attractin, will no doubt help to elucidate its function. Is there any role for αMSH in the immune system and might attractin be involved in that? Certainly αMSH has anti-inflammatory activity in animals. At least two cell types involved in inflammation express MC1R: neutrophils, whose chemotaxis to sites of inflammation might be inhibited by αMSH; and macrophages, which are inhibited by αMSH from releasing nitric oxide, a mediator of inflammation[20,21]. These processes are rather different from those described that involve attractin *in vitro*, but it will nevertheless be useful to investigate the inflammatory response of mahogany mice.

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Genomic imprinting in mammals
an interplay between chromatin and DNA methylation?

Most imprinted loci have key regulatory elements that are methylated on only one of the parental chromosomes. For several of these 'differentially methylated regions', recent studies establish that the unmethylated chromosome has a specialized chromatin organization that is characterized by nuclease hypersensitivity. The novel data raise the question of whether specific proteins and associated chromatin features regulate the allele-specificity of DNA methylation at these imprinting control elements.

In mammals, the maternal and paternal genomes are both required for normal embryonic and postnatal development. Their functional non-equivalence is mediated by genomic imprinting, an epigenetic mechanism by which the expression of certain genes becomes dependent on their parental origin[1]. To date, some 30 imprinted genes have been identified in humans and mice. Many of these play key roles in growth and differentiation, and imprinting is now recognized to be an important factor in several inherited diseases and carcinogenesis in humans[2,3]. Although the precise mechanisms that allow cells to distinguish the parental chromosomes at imprinted loci are poorly understood, one epigenetic feature that is consistently associated with imprinting is CpG methylation[4]. Almost all imprinted genes have sequence elements that are methylated only on one of the two parental alleles. These are usually referred to as 'differentially methylated regions' (DMRs). Experiments involving the targeted deletion of the main mouse methyltransferase gene (*Dnmt1*), which led to loss of imprinting in mice that were deficient in cytosine methylation[5,6], imply that this methylation mark is essential for the maintenance of imprinting.

Differentially methylated regions

More recently, homologous-recombination experiments in the mouse have demonstrated that individual DMRs are, indeed, important for the expression of imprinted genes. For example, a DMR located upstream of the mouse *H19* gene[7] that is methylated on the paternal chromosome (Fig. 1) was shown to be essential for the imprinted expression both of *H19* and of the neighbouring, paternally expressed gene that encodes insulin-like growth

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factor-2 (*Igf2*)[8]. In particular, the deletion of this DMR on the maternal chromosome led to de-repression of the maternal *Igf2* gene. Based on this finding and on transgenic experiments, it has been proposed that, on the unmethylated maternal chromosome, the *H19* upstream DMR acts as a chromatin boundary that insulates *Igf2* from its enhancer sequences[3,8]. Another well-characterized imprinted mouse gene, the *Igf2*-receptor gene (*Igf2r*) on chromosome 17, is also expressed from the maternal chromosome and has an intronic DMR that is methylated exclusively on the maternal chromosome[9]. Deletion of this DMR (also referred to as 'region 2') from a yeast artificial chromosome- (YAC-)based transgene construct demonstrates that this element is essential for *Igf2r* imprinting, apparently by regulating the expression of antisense transcripts from the paternal chromosome[10]. Its allelic methylation, and that of the *H19* DMR, originates from the germ line and appears to be maintained throughout development[9]. DMRs at other imprinted mouse genes have also been shown to be established from the germ line and maintained somatically. These include the DMR in the *U2af1-rs1* gene[11] on mouse chromosome 11, and the DMR in the promoter and exon-1 of the paternally expressed *Snrpn* gene[12], both of which code for an RNA-splicing factor. A targeted deletion that comprised the DMR in the 5′-region of *Snrpn* led to the deregulation of paternally expressed genes in the entire *Snrpn* imprinted domain, probably by interfering with the establishment of imprints in the male germ line[13]. In humans, *SNRPN* (and neighbouring genes in the imprinted domain) maps to the region of the Prader–Willi and Angelman syndromes (two distinct neurodevelopmental disorders) on chromosome 15q11–q13, and is expressed only from the paternal chromosome. The finding that the 5′-region DMR is deleted in Prader–Willi syndrome patients, and that this is associated with deregulation of imprinting in the entire imprinted domain, confirms the functional importance of these 'imprinting control' sequences[14].

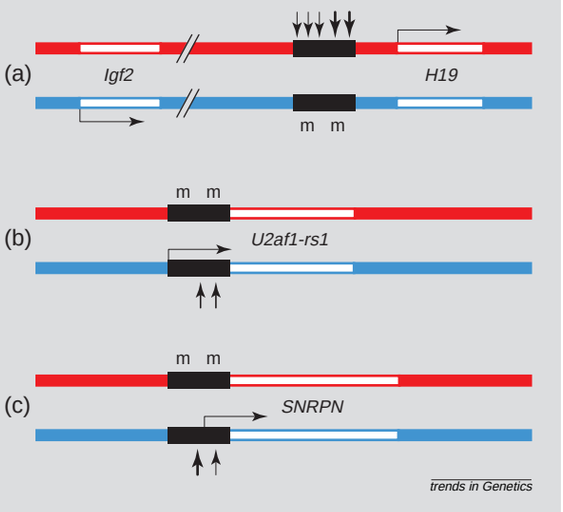
Together, the genetic studies highlight the importance of DMRs in regulating the expression of imprinted genes. In addition, the recent methylation studies have shown that the allelic-methylation patterns of these and some other DMRs derive from the germ line and appear to be maintained throughout development[1–3]. Apart from the key issue of how these methylation marks become established in either the egg or in sperm[1], these data raise the question of how allelic-methylation patterns are maintained somatically. Equally important is what prevents the unmethylated allele from becoming methylated in the germ line, and what mechanism(s) protect it from *de novo* methylation throughout development. Although definitive answers to these questions await elucidation, new insights have emerged from studies that demonstrate parental chromosome-specific organization of chromatin at DMRs. This article discusses these novel data, and proposes that specific protein factors are involved in the germ line establishment and the somatic maintenance of the allelic methylation at DMRs that might also play a role in their establishment in the germ line as well.

Interplay between chromatin and DNA methylation

In three *in vivo* chromatin studies[15–17], nuclease-hypersensitive sites were detected in the DMR upstream of the mouse *H19* gene. Five DNase-I-hypersensitive sites (and hypersensitivity to different restriction endonucleases) were detected on the unmethylated maternal chromosome, but not on the methylated paternal chromosome (Fig. 1). These hypersensitive sites were apparent in all embryonic and adult material analyzed, and also in tissues that do not express the *H19* (and the neighbouring *Igf2*) gene. A typical DNase-I-hypersensitive site corresponds to a region where nucleosomes are absent or partially disrupted. This lack of canonical nucleosomes is due to the binding of non-histone proteins. Usually, a variety of different proteins are bound, some of which are responsible for the observed hypersensitivity[18]. A specialized chromatin organization seems to be present at the *H19* DMR as well. Micrococcal-nuclease-digestion assays revealed a distinct, non-nucleosomal organization of chromatin on the maternal chromosome, precisely in the region of DNase-I hypersensitivity[17]. These results suggest that non-histone proteins are associated with the maternal copy of the DMR and that they are involved in its chromatin-boundary function. The finding that this element acts as a silencer when introduced into *Drosophila* (which has no CpG methylation) also contributes evidence for the alternative chromatin organization and the association of non-histone proteins[19].

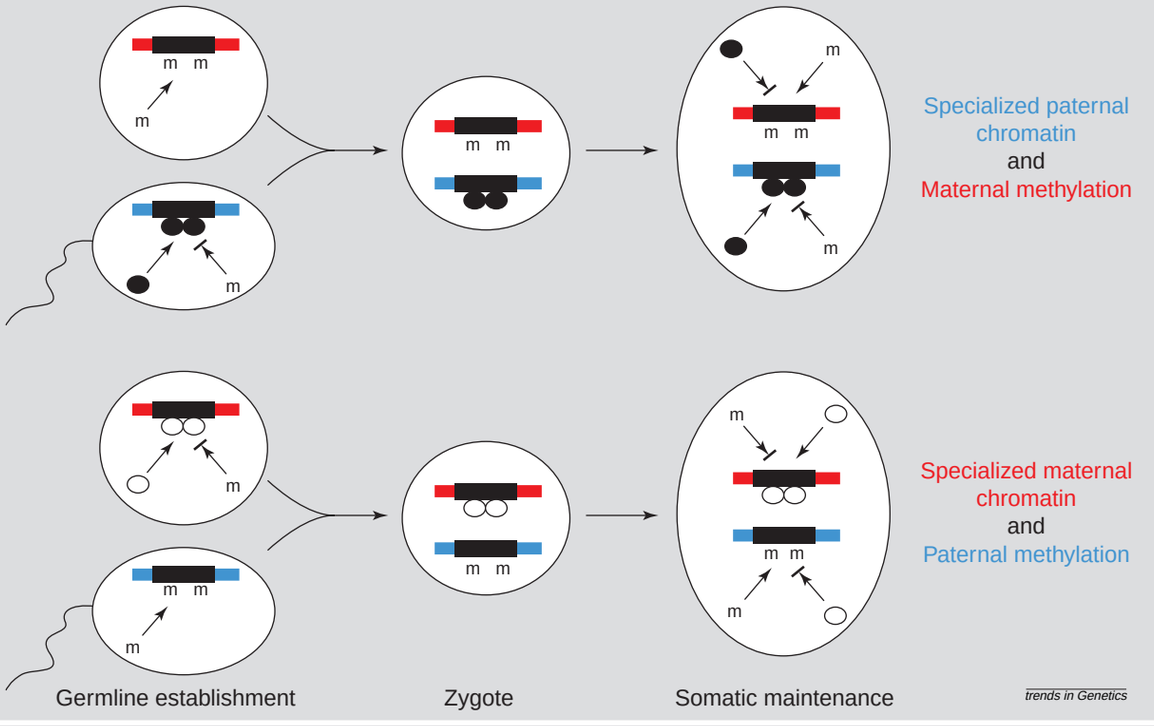
A similar allelic configuration of chromatin has been detected at the DMR in the imprinted mouse *U2af1-rs1* gene. The DMR of this paternally expressed gene has two prominent DNase-I-hypersensitive sites on the unmethylated paternal chromosome[20,21], but is rather inaccessible to nucleases on the methylated maternal chromosome[21]. Thus, both in the *H19* and in the *U2af1-rs1* DMRs, a specialized organization of chromatin, characterized by DNase-I hypersensitivity, is present only on the unmethylated chromosome. This seems to indicate that DNase-I

FIGURE 1. Nuclease hypersensitivity and DNA methylation in differentially methylated regions



In the differentially methylated regions (DMRs) shown (black boxes), allelic methylation originates from the germ line and appears to be maintained throughout development. (a) The *H19* upstream DMR (mouse), (b) the *U2af1-rs1* DMR (mouse) and (c) the *SNRPN* 5′-region DMR (human). Paternal chromosomes are in blue; maternal chromosomes in red. DNase I-hypersensitive sites are depicted as vertical arrows, methylation as 'm m', and genes as open boxes (horizontal arrows indicate transcription).

FIGURE 2. Model of mutual exclusion



In most imprinted loci there are differentially methylated regions (DMRs; black boxes) that acquire their parental chromosome-specific methylation (m m) in either the male or the female germ line. The model proposes that the unmethylated parental allele is protected from methylation because of the binding of specific proteins (filled or open ovals) causing the formation of specialized chromatin organization. Protein factors are not necessarily identical for different DMRs, also not for DMRs that are unmethylated on the same parental genome. Conversely, methylation at the methylated parental allele and associated chromatin features[21,24] prevent the binding of such factors. These two competing mechanisms are involved in the maintenance of the maternal and paternal epigenotypes during development, and could be involved in their establishment in the germ lines as well (see text).

hypersensitivity and DNA methylation represent alternative epigenotypes that are mutually exclusive. The finding that alterations in the one affect the other indicates that the two epigenotypes are, indeed, somehow interdependent. Hence, in the *H19* DMR, the loss of hypersensitivity might occur when embryonic stem cells (ES) are cultured *in vitro*, and is associated with a gain of methylation[17]. Conversely, in the *U2af1-rs1* DMR, the loss of DNA methylation is consistently associated with a gain of hypersensitivity[21].

For DMRs in the human *SNRPN* locus, recent data provide evidence for the alternative organization of chromatin at the unmethylated allele, which is characterized by nuclease hypersensitivity[22]. Analysis of DNase-I (and restriction-endonuclease) sensitivity throughout the *SNRPN* transcription unit revealed several sites of parental chromosome-specific hypersensitivity[22]. The most prominent of these colocalizes with the DMR that is located in the 5′-portion of *SNRPN*. In this 'imprinting-control centre', two strong DNase-I sites are present on the unmethylated paternal chromosome, but not on the methylated maternal chromosome (Fig. 1). The recent finding[23] that the *SNRPN* DMR (and similarly the *H19* DMR) functions as a classical silencer in transgenic *Drosophila* also suggests that this element has specialized chromatin features and possibly interacts with non-histone proteins.

Based on the new chromatin data, we propose that, during gametogenesis, the decision is made between methylation of the DNA or the establishment of a chromatin organization that is characterized by nuclease hypersensitivity. Non-histone factors that promote a specialized chromatin organization might be available in only one of the two germ lines, and once such an organization is achieved, this would protect the regulatory element from DNA methylation in the germ line and during development. Conversely, if such specific non-histone proteins are absent in the germ line (and factors to methylate the DMR are available), the sequence element becomes methylated. After fertilization, this DNA methylation and its associated chromatin features[24] would not allow the binding of non-histone proteins or the formation of a non-canonical chromatin organization. The essential component of the proposed model is that, in somatic cells, specific proteins are associated with the unmethylated allele of DMRs and prevent the DNA from becoming methylated. Some of these specific factors associated with DMRs are also present in only one of the two germ lines and would thus specify the parental allele that remains unmethylated. The validity of this model of mutual exclusion (Fig. 2) requires further testing in somatic and germ cells.

Perspectives

It is notable that, similar to the differential DNA methylation, the parental chromosome-specific nuclease hypersensitivity in the *H19*, *U2af1-rs1* and *SNRPN* genes is present in all tissues analysed. This suggests that at least some

of the associated factors are constitutive and could be involved in preventing the DMR from becoming methylated during development. Such a role would be most pertinent during pre-gastrulation stages, when the global wave of *de novo* methylation occurs[6]. Significantly, for the *H19* and *U2af1-rs1* DMRs, it has been established that their allelic hypersensitivity is also present in ES cells[17,21], which are approximately equivalent to the inner cell mass of a blastocyst.

From the current data it is unclear whether cytosine methylation and non-histone protein binding are mutually exclusive in the male and female germ line, and whether this confers the allele-specificity of DNA methylation at DMRs. However, several studies indicate that there could be specific proteins in developing germ cells that mediate the methylation status of DMRs. Birger *et al.*[25] have shown recently that the intronic DMR of the mouse *Igf2r* gene contains a sequence element that, after its injection into the female pronucleus of fertilized eggs, becomes methylated during pre-implantation development. By contrast, the same element injected into the male pronucleus remains protected from methylation, at least until the blastocyst stage. Based also on their *in vitro* band-shift assays, the authors suggest that there is an allele-discriminating protein that is associated specifically with the unmethylated paternal chromosome at this DMR (Ref. 25). In another study, on the imprinted mouse *U2af1-rs1* gene, Hatada and co-workers reported that methylation at the endogenous *U2af1-rs1* locus can be affected by the presence of multiple copies of its own transgene in the testis[26]. Frequently, offspring (even those that were non-transgenic) of hemizygous transgenic males acquired full methylation on the (normally unmethylated) paternal copy of the *U2af1-rs1* gene, in addition to the methylation on the maternal copy. An explanation for this intriguing finding could be that there are specific factor(s) in the male germ line that associate with the *U2af1-rs1* gene and keep it unmethylated[26]. Competing, additional transgene copies of their recognition elements (perhaps the paternal hypersensitive sites) would interfere with the binding of these protein(s) to the endogenous gene.

The challenge now is to determine which non-histone protein factors are associated with DMRs. As of yet, there are no indications about their possible nature. However, although non-homologous, the three DMRs in which allelic nuclease hypersensitivity has been identified are all rather G+C-rich. Apart from their allelic methylation, they would, in fact, resemble CpG islands: CpG-rich sequences that are usually associated with the 5′ domains of housekeeping genes and certain tissue-specific genes. It has been shown that CpG islands have an alternative chromatin structure, with nucleosome-free regions, and various *in vivo* studies demonstrate their association with proteins[27]. Because CpG islands are normally maintained in an unmethylated state, it has been proposed that specific proteins are involved in protecting CpG island sequences from *de novo* methylation during development[27]. A number of studies indicate that members of the SP1 family[28,29], and other proteins, including NF-κB (Refs 30, 31), could fulfil such protective roles. How their precise actions come about, and whether these factors can specify sites of (passive or active) demethylation at specific developmental stages[30–32], remains to be resolved. Given the similarities between CpG islands and DMRs, it should be interesting to determine whether functionally related proteins are involved in the regulation of parental allele-specific DNA methylation. The fact that the DMRs discussed do not share significant sequence homology suggests that different proteins are involved; these could, however, be structurally similar and part of a family.

The novel chromatin studies do not address an other important question, that of what protects the methylated parental allele against demethylation. The DMRs discussed here retain their allele-specific DNA methylation during the genome-wide wave of demethylation that occurs during pre-implantation development[6], and thereafter, and this suggests the involvement of one or more protective mechanisms. The model of mutual exclusion does not preclude the involvement of specific factors and chromatin modifications also on the methylated parental chromosome. In fact, dependent on its density, CpG methylation might be providing an alternative type of chromatin at the methylated chromosome[24,33]. In this regard, one possibility would be the association of specific 'methylated-DNA-binding proteins' (MeCPs) to methylated cytosine residues. Recent studies have shown that a member of this family, MeCP2, recruits a multi-protein complex that includes histone deacetylases[33,34]. This deacetylase activity can decrease local levels of core histone acetylation and, presumably, thereby alter chromatin conformation[24]. Relative to the scenarios of chromatin modification associated with CpG methylation, it is interesting to note that, on the methylated chromosome, the entire mouse *U2af1-rs1* locus was shown to be highly resistant to DNase-I and restriction endonucleases, which suggests a compacted chromatin conformation[21]. Future research will tell whether the conformational features of chromatin at imprinted loci are associated with histone modifications and protein binding specifically at the methylated allele.

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Copying out our ABCs
the role of gene redundancy in interpreting genetic hierarchies

The complete sequence of the *Arabidopsis* genome is scheduled to be determined by the end of the year 2000. While this goal could prove to be something of a moving target (the estimated size of the genome has grown from 120 Mb to 130 Mb over the last year[1]), it is clear that the majority of genes required for higher plant growth, reproduction and development will have been described within this time frame. Some of the implications of this landmark achievement are already becoming clear, even though less than a half of the genome has been sequenced.

Arabidopsis has one of the most simplified plant genomes, with only limited evidence for segmental duplications, little repetitive DNA, and with good diploid genetics. One of the most important observations to emerge from genome studies, however, is that most *Arabidopsis* genes are not unique. Of 100 genes found between *prolifera* and *GA1* on chromosome 4, for example, 65 are members of small multigene families. Based on these and other data emerging from the genome project, at least two-thirds of *Arabidopsis* genes have one or more closely related homologs (L. Parnell and W.R. McCombie, pers. commun.). This is especially true of key regulatory molecules such as transcription factors, receptor kinases, F-box proteins and cell-cycle regulators. For example, the MADS box gene family has at least 50 members, while there might be more than 300 receptor kinases. What are the implications of this widespread gene duplication?

It is possible that gene duplications have allowed each family member to evolve a unique function, for example in a specialized cell type. However, in many cases, family members have overlapping expression domains and so might effect the same process in the same cell type. With the advent of the polymerase chain reaction, it has become a relatively trivial matter to obtain insertional mutants in such genes via site-selected mutagenesis. The major conclusion emerging from studies of this sort is surprising: most insertional mutants have no discernible phenotype. Sometimes, the corresponding gene might be non-functional, representing a pseudogene or other evolutionary relic. However, genetrap studies reveal that many expressed genes, when disrupted, can still lack a detectable phenotype[2]. Mutations in such genes might not be recovered because they have subtle or conditional phenotypes. Alternatively, such mutations might not be recovered because multiple closely related genes encode that function. In such cases, double, triple and sometimes even more redundant combinations of mutations might be required to reveal a mutant phenotype.

What might the consequences of this redundancy be for developmental genetics? One important ramification is in the ordering of regulatory pathways by double-mutant analyses. As any genetics textbook will point out, if single-mutant phenotypes are distinct and the double mutant resembles one of the single mutants, then the mutations are generally interpreted to affect steps in a linear pathway. If the double mutant shows an additive phenotype, then the two mutations are thought to affect separate, unrelated processes. Alternatively, the double mutant

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